



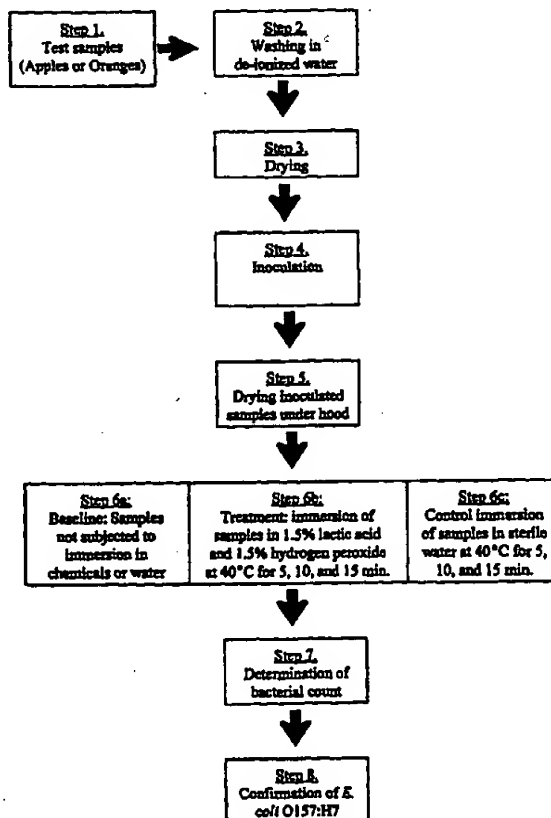
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A23L 3/34</b>		<b>A1</b>	(11) International Publication Number: <b>WO 99/44444</b>
			(43) International Publication Date: 10 September 1999 (10.09.99)
(21) International Application Number: <b>PCT/US99/04586</b> (22) International Filing Date: <b>2 March 1999 (02.03.99)</b> (30) Priority Data: <b>60/076,487</b> <b>2 March 1998 (02.03.98)</b> <b>US</b> (71) Applicant: <b>THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; DW Brooks Drive, 633 Boyd Graduate Studies Research Center, Athens, GA 30602-7411 (US).</b> (72) Inventors: <b>DOYLE, Michael, P.; 107 Stonington Drive, Peachtree City, GA 30269 (US). VENKITA-NARAYANAN, Kumar, S.; 312 Terrane Ridge, Peachtree City, GA 30269 (US).</b> (74) Agents: <b>FERBER, Donna, M. et al.; Greenlee, Winner and Sullivan, P.C., Suite 201, 5370 Manhattan Circle, Boulder, CO 80303 (US).</b>		(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report.</i> <i>With amended claims.</i>	

(54) Title: **REDUCTION OF BACTERIAL PATHOGENS ON FRESH FOOD ITEMS**

## (57) Abstract

Methods and compositions for treating uncooked food items, such as produce, so as to reduce viable bacteria, especially enteropathogenic bacteria, on the surfaces of produce and other uncooked food products. Use of a solution containing lactic acid and at least one of hydrogen peroxide, sodium benzoate or glycerol monolaurate, in contact with the food item for 5 sec to 30 min. effectively reduces certain food-borne pathogens, especially *Escherichia coli* O157:H7. The figure is a flowchart for the fresh fruit model experiment in which known populations of *E. coli* O157:H7 were applied to apples and oranges and treated with certain washing steps, followed by determination of the bacterial cell counts.





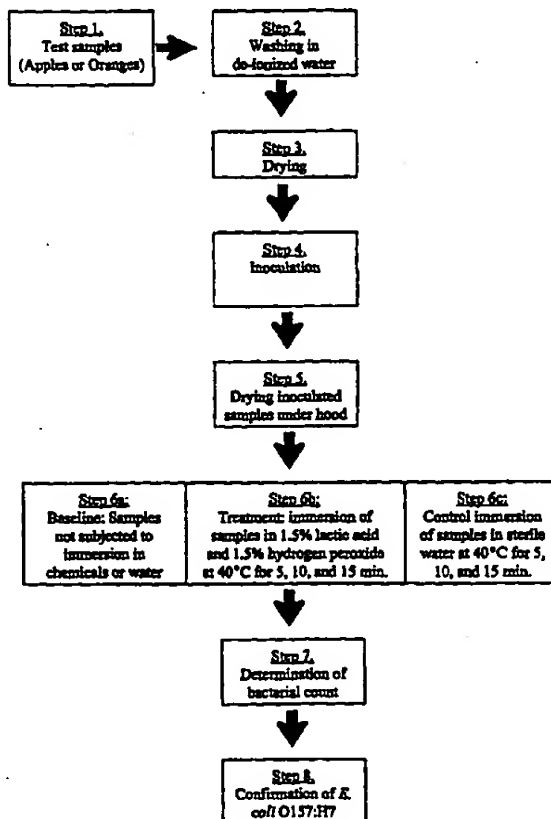
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A23L 3/34</b>		(11) International Publication Number: <b>WO 99/44444</b>
<b>A1</b>		(43) International Publication Date: 10 September 1999 (10.09.99)
(21) International Application Number: <b>PCT/US99/04586</b> (22) International Filing Date: <b>2 March 1999 (02.03.99)</b> (30) Priority Data: <b>60/076,487</b> <b>2 March 1998 (02.03.98)</b> <b>US</b> (71) Applicant: <b>THE UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; DW Brooks Drive, 633 Boyd Graduate Studies Research Center, Athens, GA 30602-7411 (US).</b> (72) Inventors: <b>DOYLE, Michael, P.; 107 Stonington Drive, Peachtree City, GA 30269 (US). VENKITA-NARAYANAN, Kumar, S.; 312 Terrane Ridge, Peachtree City, GA 30269 (US).</b> (74) Agents: <b>FERBER, Donna, M. et al.; Greenlee, Winner and Sullivan, P.C., Suite 201, 5370 Manhattan Circle, Boulder, CO 80303 (US).</b>		(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b>  Published <i>With international search report.</i> <i>With amended claims.</i>

## (54) Title: REDUCTION OF BACTERIAL PATHOGENS ON FRESH FOOD ITEMS

## (57) Abstract

Methods and compositions for treating uncooked food items, such as produce, so as to reduce viable bacteria, especially enteropathogenic bacteria, on the surfaces of produce and other uncooked food products. Use of a solution containing lactic acid and at least one of hydrogen peroxide, sodium benzoate or glycerol monolaurate, in contact with the food item for 5 sec to 30 min. effectively reduces certain food-borne pathogens, especially *Escherichia coli* O157:H7. The figure is a flowchart for the fresh fruit model experiment in which known populations of *E. coli* O157:H7 were applied to apples and oranges and treated with certain washing steps, followed by determination of the bacterial cell counts.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## REDUCTION OF BACTERIAL PATHOGENS ON FRESH FOOD ITEMS

### CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from United States Provisional Application No.  
5 60/076,487, filed March 2, 1998.

### ACKNOWLEDGMENT OF FEDERAL RESEARCH SUPPORT

This invention was made, at least in part, with funding from the United States Food  
and Drug Administration. Accordingly, the United States Government has certain rights in  
10 this invention.

### BACKGROUND OF THE INVENTION

The field of this invention is the area of food safety; in particular, the invention relates  
to compositions and methods for treatment of produce or meat products to reduce or  
inactivate bacterial pathogens on that produce or meat.

15 Reported outbreaks of food-borne illness involving fruits and vegetables have  
increased during the last decade. However, there are no effective methods currently available  
to reduce or inactivate pathogenic bacteria on produce. There is a need in the art for methods  
and compositions to be used as surface treatments to reduce or inactivate bacterial pathogens  
including, but not limited to, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and  
20 *Salmonella enteritidis* on fruits and vegetables.

*Escherichia coli* O157:H7, which causes hemorrhagic colitis and hemolytic uremic  
syndrome, has emerged as a food-borne pathogen of major public health concern [Padhye and

Doyle (1991) *Appl. Environ. Microbiol.* 57:2693-2698]. A massive outbreak of *E. coli* O157:H7 infection involving more than 9,500 cases and nine deaths occurred in Japan in 1996. A recent outbreak in the United States was associated with consumption of unpasteurized apple juice [Centers for Disease Control and Prevention (1997) *Morbidity and Mortality Weekly Rep.* 46:4-8]. A wide variety of foods including meat, milk, fruits and vegetables have been implicated as vehicles of *E. coli* O157:H7 infection.

The USDA-FSIS Pathogen Reduction final rule recommends antimicrobial treatments as a method for reducing or inactivating pathogenic bacteria in foods [USDA-FSIS (1995) Proposal Billing Code 3410-DM-P. Docket No. 93-016P.RIN 0583.AB69. USDA, Beltsville, MD]. Effective methods to reduce or eliminate pathogens in foods are important to the successful implementation of Hazard Analysis Critical Control Point (HACCP) programs by the food industry. Lactic acid is a multipurpose GRAS (Generally Regarded As Safe) chemical that has an antimicrobial effect on a variety of bacterial pathogens [Anderson and Marshall (1990) *J. Food Sci.* 55:903-905; Greer and Dilts (1992) *Food Res. Int.* 25:355-364]. However, several researchers have determined that *E. coli* O157:H7 has unusual tolerance to acidic conditions [Zhao et al. (1993) *Appl. Environ. Microbiol.* 59:2526-2530; Brackett et al. (1994) *J. Food Prot.* 57:198-203; Conner and Kotrola (1995) *Appl. Environ. Microbiol.* 61:382-385]. Despite their acid tolerance, strains of *E. coli* O157:H7 are sensitive to heat, with D values similar to those of *Salmonella* spp. [Doyle et al. (1997) In *Food Microbiology: Fundamentals and Frontiers* (Eds. Doyle, M.P., Beuchat, L.R. and Montville, T.J. pp. 171-191. American Society for Microbiology, Washington, D.C.).

The antimicrobial activity of organic acids can be increased or potentiated when combined with other food preservatives [Bøgh-Sørensen (1994) In *Food Preservation by Combined Processes* (Eds. Leistner, L. and Gorris, L.G.M.) pp. 17-18. Food Linked Agro-Industrial Research Concerted Action No.7, Subgroup B. The Netherlands; Marshall and Kim (1996) *J. Food Quality* 19:317-329] or heat [Greer and Dilts (1992) *Food Res. Int.* 25:355-364; Smulders et al. (1986) *J. Food Technol.* 21:419-436]. Many GRAS chemicals, including sodium benzoate [Zhao et al. (1993) *Appl. Environ. Microbiol.* 59:2526-2530], sodium nitrite and potassium sorbate [Tsai and Chou (1996) *J. Sci. Food Agric.* 71:10-12],

and trisodium phosphate [Kim and Slavik (1994) *J. Food Sci.* 59:20-22], have been reported to possess antibacterial activity against *E. coli* O157:H7. However, these chemicals are not entirely effective in eliminating or inactivating the pathogen.

There is a longfelt need in the art for improved food safety, especially as related to surface contamination of fresh food items including, but not limited to, fresh fruits and vegetables. Surface contamination of such foods eaten raw has resulted in life-threatening illnesses, for example, when contaminated with viable *E. coli* O157:H7 cells.

### SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods and compositions effective for inactivating or reducing the number of bacteria on the surfaces of produce and other uncooked food items. In particular, the compositions and methods of the present invention reduce the numbers of pathogenic bacteria on the surfaces of fresh fruits and vegetables, especially by inactivating enteric pathogens including, but not limited to, *Escherichia coli* strains such as those of O157:H7, *Salmonella spp*, *Listeria monocytogenes*, *Campylobacter*, *Yersinia*, *Vibrio* species, including without limitation, *cholerae*, *parahaemolyticus* and *vulnificus*. The method of the present invention is to wash or soak the fresh food items in a solution comprising lactic acid and either hydrogen peroxide, sodium benzoate or glycerol monolaurate, under conditions of time (30 sec to 30 min) and temperature (5°C to 70°C) such that numbers of at least one bacterial pathogen are reduced. Optimization of conditions allows for maximum impact on bacterial number and minimal impact on food taste, texture and visual qualities. Desirably the pH of the solution is below 3.5, preferably from about 0.5 to about 3.5. A suitable composition comprises 1% to 3% acid, desirably 1.5%, lactic acid plus 0.1% to 3% hydrogen peroxide or 0.05% to 1.0%, desirably 0.1%, sodium benzoate, or 0.001% to 0.05%, desirably 0.005% glycerol monolaurate. The lactic acid in the wash or soak solution can be substituted by one or more of acetic, malic, propionic, tartaric, mandelic and phosphoric acid, at similar concentration and pH.

It is a further object of this invention to provide methods and compositions for

reducing numbers of or inactivating bacteria, especially pathogens, on the surfaces of fish, shellfish, raw cut meat products and carcasses. Pathogens which are inactivated or whose numbers are reduced include, without limitation, species of *Salmonella*, *Yersinia*, *Listeria*, *Campylobacter* and *Escherichia*, especially *E. coli* O157:H7, and *Vibrio* species, including without limitation, *cholerae*, *parahemolyticus* and *vulnificus*. Fish, shellfish, meat carcasses (hog, beef, lamb, buffalo and the like) are washed with or immersed in a solution comprising an acid, especially lactic acid, and either hydrogen peroxide, sodium benzoate or glycerol monolaurate, under conditions of time (30 sec to 30 min) and temperature (4°C to 70°C) such that numbers of at least one bacterial pathogen are reduced. Optimization of conditions allows for maximum impact on bacterial number and minimal impact on food taste, texture and visual qualities. Desirably the pH of the solution is below 3.5, preferably from about 0.5 to about 3.5. A suitable composition comprises 1% to 1.5% lactic acid plus a second component: 0.1% to 2% hydrogen peroxide, 0.1% sodium benzoate or 0.005% glycerol monolaurate. The lactic acid in the wash or soak solution can be substituted by one or more of acetic, malic, propionic, tartaric, mandelic and phosphoric acid, at similar concentration and pH.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the inactivation of *E. coli* O157:H7 in 0.1% peptone containing 1.0 or 1.5% lactic acid plus 0.1% hydrogen peroxide at 8°C, 22°C and 40°C. Arrow indicates that *E. coli* O157:H7 was not detected at the minimum level of sensitivity (< 10 CFU/ml).

Figure 2 shows the inactivation of *E. coli* O157:H7 in 0.1% peptone containing 1.0 or 1.5% lactic acid plus 0.1% sodium benzoate at 8°C, 22°C and 40°C. Arrow indicates that *E. coli* O157:H7 was not detected at the minimum level of sensitivity (< 10 CFU/ml).

Figure 3 depicts the inactivation of *E. coli* O157:H7 in 0.1% peptone containing 1.0 or 1.5% lactic acid plus 0.005% glycerol monolaurate at 8°C, 22°C and 40°C. Arrow indicates that *E. coli* O157:H7 was not detected at the minimum level of sensitivity (< 10 CFU/ml).

Figures 4A-4C demonstrate the inactivation of *E. coli* O157:H7 in 0.1% peptone containing 1.5% lactic acid, 0.1% hydrogen peroxide, 0.1% sodium benzoate or 0.005% glycerol monolaurate at 40°C (Fig. 4A), 22°C (Fig. 4B) and 8°C (Fig. 4C).

Figure 5 is a flowchart for the fresh fruit model experiment in which known populations of *E. coli* O157:H7 were applied to apples and oranges and treated with certain washing steps, followed by determination of the bacterial cell counts.

Figure 6 illustrates where apples were inoculated with *E. coli* O157:H7 in the experiment outlined in Fig. 5.

#### DETAILED DESCRIPTION OF THE INVENTION

Reported outbreaks of food-borne illness involving *Escherichia coli* O157:H7 have increased in the United States during the last decade, with a variety of food products being implicated as vehicles of infection. Meats, including beef, hamburger and chicken have been shown to contain bacterial pathogens including, without limitation, *E. coli* O157:H7, *Salmonella spp.*, *Listeria monocytogenes* and *Campylobacter spp.* Fish and shellfish may be contaminated with *Vibrio* species including, but not limited to, *cholerae*, *parahaemolyticus* and *vulnificus*. Additionally, fresh fruits and vegetables and unpasteurized fruit juice products have also been shown to contain dangerous bacterial pathogens and have been linked to enteric disease outbreaks. The young, the elderly and those with compromised immune systems are especially vulnerable to infection. Unless effective means are developed to reduce bacterial numbers on food products, food-borne infections are likely to become an increasing problem as the population of this country ages.

With the goal of effective treatments of contaminated produce to reduce numbers of pathogenic bacteria, the present inventors carried out studies to determine the efficacy of combinations of various GRAS chemicals and moderate temperatures to kill *E. coli* O157:H7, chosen as a model for enteric bacterial pathogens. A five-strain mixture of *E. coli* O157:H7 of approximately  $10^6$  CFU/ml was inoculated into 0.1% peptone solutions containing 1.0 or



1.5% lactic acid plus 0.1% to 2% hydrogen peroxide, 0.1% sodium benzoate or 0.005% glycerol monolaurate. The solutions were incubated at 8°C for 0, 15 and 30 min; at 22°C for 0, 10 and 20 min; or at 40°C for 0, 10 and 15 min, and populations of *E. coli* O157:H7 were determined at each sampling time.

5        The pH of the different combinations of treatments ranged from 2.75-3.04, with the control (0.1% peptone) of pH 7.05 (Table 1). Treatments containing lactic acid and hydrogen peroxide had the lowest pH (2.75-2.88), followed by those containing lactic acid and glycerol monolaurate (pH 2.81-2.91), and lactic acid and sodium benzoate (pH 2.93-3.04).

10        The effect of 1.0 or 1.5% lactic acid plus 0.1% hydrogen peroxide at 8°, 22° and 40°C on the five-strain mixture of *E. coli* O157:H7 is illustrated in Figure 1. *E. coli* O157:H7 populations declined to undetectable levels (as determined by both plating and enrichment procedures) after 10 min of exposure to 1.0 and 1.5% lactic acid plus 0.1% hydrogen peroxide at 40°C, and after 20 min of exposure to 1.5% lactic acid plus 0.1% hydrogen peroxide at 22°C. However only a 2.5 log *E. coli* O157:H7/ml reduction occurred in 1.5% lactic acid plus 0.1% hydrogen peroxide held for 30 min at 8°C.

15        The inactivation of *E. coli* O157:H7 in 0.1% peptone containing 1.0 or 1.5% lactic acid plus 0.1% sodium benzoate at 8°, 22° and 40°C is depicted in Figure 2. In the presence of 1.5% lactic acid plus 0.1% sodium benzoate, *E. coli* O157:H7 populations were reduced to undetectable levels (as determined by both plating and enrichment procedures) after 15 min at 40°C, whereas a reduction of only 2.0 to 2.5 log *E. coli* O157:H7/ml was observed at 30 min at 8°C, and at 20 min at 22°C, respectively.

20        In the treatment containing 1.5% lactic acid plus 0.005% glycerol monolaurate, *E. coli* O157:H7 populations were reduced by 2.0 log CFU/ml in 30 min, > 5.0 log CFU/ml in 20 min, and to undetectable levels in 15 min, at 8°, 22° and 40°C, respectively (Figure 3).

25        Selective culture media can inhibit the recovery of heat- and acid-stressed cells of *E. coli* O157:H7 [Abdul-Raouf et al. (1993) *Appl. Environ. Microbiol.* 59:2364-2368; Clavero

and Beuchat (1996) *Appl. Environ. Microbiol.* 62:2735-2740]. For example, Sorbitol MacConkey agar (SMA), when used as an enumeration medium, failed to recover *E. coli* O157:H7 cells after they were subjected to stress by heat and acids. Hence, in this study, TSA and TSB were used as the media for enumeration and enrichment, respectively.

5           Lactic acid is biocidal to a wide range of microorganisms; however, *E. coli* O157:H7 is unusually tolerant to its antimicrobial properties. Conner and Kotrola [(1995) *Appl. Environ. Microbiol.* 61:382-385] determined that *E. coli* O157:H7 was able to survive for up to 56 days in TSB acidified to pH 4.7 with lactic acid. Similarly, Abdul-Raouf et al. [(1993) *Appl. Environ. Microbiol.* 59:2364-2368] determined that *E. coli* O157:H7 survived well in  
10   beef slurries with lactic acid. Our studies with lactic acid (1.5%), hydrogen peroxide (0.1%), sodium benzoate (0.1%), and glycerol monolaurate (0.005%) as sole treatments on *E. coli* O157:H7 in 0.1% peptone water revealed that none of the chemicals could reduce *E. coli* O157:H7 populations by 5.0 log CFU/ml (Figure 4A, 4B, 4C). Of the four chemicals evaluated, lactic acid had the greatest biocidal effect, reducing *E. coli* O157:H7 counts by  
15   approximately 1.3 log CFU/ml, 2.0 log CFU/ml, and 4.0 log CFU/ml after 30 min of exposure at 8°, 20 min at 22° and 10 min at 40°C, respectively. No substantial reduction (< ca. 1.0 log CFU/ml) of *E. coli* O157:H7 occurred in treatments of 0.1% hydrogen peroxide, 0.1% sodium benzoate, or 0.005% glycerol monolaurate.

          Of the combinations of treatments used in this study, lactic acid plus hydrogen  
20   peroxide was most effective in reducing *E. coli* O157:H7 populations. At 8°C, the maximum reduction in *E. coli* O157:H7 populations was about 2.5 log CFU/ml, achieved by treatment with lactic acid plus hydrogen peroxide. The combination of 1.5% lactic acid and 0.1% hydrogen peroxide had the greatest antimicrobial activity, with > 6.0 log *E. coli* O157:H7/ml reduction occurring within the shortest exposure time, at both 22° and 40°C. Hydrogen  
25   peroxide is a GRAS approved chemical level for use as an antimicrobial agent in cheese making and whey processing at the 0.05% level [Davidson et al. (1983) In Naturally Occurring and Miscellaneous Food Antimicrobials. (Eds. Brannen, A.L. and Davidson, M.P.) pp. 385-391. Marcel Dekker, Inc., New York]. A treatment of milk with 0.06% hydrogen peroxide at 130°F for 1 min was reported to be biocidal to *Clostridium* spp. and *Bacillus* spp.

[Nielsen (1987) *Am. Dairy Ref.* 39(6):32-34]. *E. coli* populations on chicken carcasses were reduced significantly by 5,300 ppm of hydrogen peroxide in chiller water [Lillard and Thomson (1983) *J. Food Sci.* 48:125-126]. However, the efficacy of 0.1% hydrogen peroxide in inactivating *E. coli* O157:H7 within 30 min at 8°, 20 min at 22° or 10 min at 40°C is minimal. The addition of 1.0% lactic acid in combination with 0.1% hydrogen peroxide greatly enhances the antimicrobial activity of the two chemicals.

In a model fruit experiment, known numbers ( $\sim 5 \times 10^7$ ) of *E. coli* O157:H7 were applied to the stem ends of oranges and apples, and the fruits were allowed to dry. The average population of *E. coli* O157:H7 recovered from apples after inoculation was 7.0 log CFU/apple. Immersion of apples in water containing 1.5% lactic acid plus 1.5% hydrogen peroxide at 40°C for 5, 10 or 15 min. resulted in complete inactivation of *E. coli* O157:H7 (<5 log CFU/apple). However, control apples immersed in sterile deionized water at 40°C for the same durations yielded a mean *E. coli* O157:H7 population of 5.4 log CFU/apple. Further, an average count of 4.5 log CFU/ml of the pathogen was recovered from the deionized water in which the inoculated apples were submerged, whereas, no *E. coli* O157:H7 could be detected in the treatment water containing lactic acid and hydrogen peroxide (as determined by both plating and enrichment procedures).

In the case of oranges, the mean inoculation level was 7.3 log CFU/orange. Oranges immersed in water containing 1.5% lactic acid plus 1.5% hydrogen peroxide at 40°C for 10 or 15 min. yielded a mean *E. coli* O157:H7 population of 1.5 log CFU and < 1.0 log CFU/apple (detected by enrichment only), respectively. However, control apples subjected to immersion in deionized water yielded a bacterial count of > 5.5 log CFU/orange after 10 and 15 min. exposure times. As observed in the studies with apples, no *E. coli* O157:H7 survived in the treatment solution after immersion of oranges, whereas, a mean *E. coli* O157:H7 count of 4.5 log CFU/ml was recovered from the deionized water. See Tables 1 and 2 for a summary of these results.

Immersion of apples and oranges in water containing 1.5% lactic acid plus 1.5% hydrogen peroxide at 40°C was very effective in reducing *E. coli* O157:H7 on the fruits.

Although immersion of fruits in water itself reduced the population of *E. coli* O157:H7 by 1 to 1.5 log CFU, a significant population of the pathogen survived in the water, representing a potential source of recontamination or cross contamination, in case the same water is used for soaking or washing fruits.

- 5           The exemplified wash solution (1.5% lactic acid, 1.5% hydrogen peroxide similarly reduces numbers of bacterial pathogens on fresh food items (e.g., raw fruits and vegetables) including, but not limited to, *Escherichia coli* including O157:H7, *Salmonella* spp., *Vibrio* spp., *Shigella* spp. and *Listeria* spp.

- 10           Glycerol monolaurate (monolaurin) is a food-grade chemical approved by the Food and Drug Administration as an emulsifier (21 CFR GRAS 182.4505). Besides its emulsification property, glycerol monolaurate exhibits antimicrobial activity against many spoilage and pathogenic microorganisms [Kabara (1979) *J. Am. Oil Chem. Soc.* 56:760A-767A; Beuchat (1980) *Appl. Environ. Microbiol.* 39:1178-1182; Kato, N. (1981) *J. Food Safety* 3:12-15]. Gram-positive bacteria are more sensitive to the antimicrobial effects of  
15           monolaurin than gram-negative bacteria [Monk et al. (1996) *J. Appl. Bacteriol.* 81:7-18]. The antimicrobial activity of glycerol monolaurate is pH dependent and can be increased by decreasing the pH of the medium [Kato and Shibasaki (1976) *J. Antibacterial. Antifung. Agents* 4:254-261; Oh and Marshall (1992) *J. Food Prot.* 55:449-450]. The combination of  
20           lactic acid (1.0 and 1.5%) and monolaurin (0.005%) inactivated > 6.0 log *E. coli* O157:H7/ml at 40°C in 15 min. At 22°C, this treatment reduced *E. coli* O157:H7 by approximately 5 log CFU/ml in 20 minutes. The antimicrobial activity of monolaurin is attributed to its effect on bacterial cell membrane permeability resulting in inhibition of synthesis of macromolecules [Galibraith and Miller (1973) *J. Appl. Bacteriol.* 36:659-675; Vadehra et al. (1985) In *The Pharmacological Effect of Lipids II* (Ed. Kabara, J.J.) pp. 89-95. American Oil Chemists  
25           Society, Champaign, IL]. Reducing the pH of the suspending medium increases the uptake of monolaurin by bacterial cells [Oh and Marshall (1992) *J. Food Prot.* 55:449-450].

Although treatment containing lactic acid and sodium benzoate was less effective against *E. coli* O157:H7 than the other two treatments studied, > 6.0 log CFU/ml inactivation

of the pathogen was observed in 1.5% lactic acid plus 0.1% sodium benzoate held at 40°C for 15 min. *E. coli* O157:H7 populations were reduced by only 2.0 to 2.5 log CFU/ml after 20 min at 22°C and 30 min at 8°C, respectively.

5 Temperature was a critical factor influencing antimicrobial activity of the different treatments on *E. coli* O157:H7. All three combinations of the GRAS chemicals evaluated were effective in inactivating > 6.0 log *E. coli* O157:H7/ml at 40°C for 15 min. At 8°C, treatments for up to 30 min could only reduce < 3.0 log *E. coli* O157:H7/ml. This study also indicated that a combination of lactic acid with hydrogen peroxide, sodium benzoate or glycerol monolaurate was more inhibitory to *E. coli* O157:H7 than when they were present  
10 individually. Based on the retail price of the chemicals determined to be effective, the combinations and concentrations of chemicals reported herein are practical and can be applied as surface treatments to inactivate *E. coli* O157:H7 on suitable raw agricultural commodities.

The above combination treatments used in media tests or as produce washes or soaks, desirably at a solution temperature between about 8 and about 70°C, preferably between  
15 about 20 and about 45°C, and for a time from about 2 to about 30 minutes are effective in reducing or inactivating *Salmonella*, *Yersinia*, *Campylobacter*, *Listeria*, *Vibrio* species, as well as *E. coli* O157:H7 or other species or strains of *Escherichia*.

While lactic acid at a concentration of 1 to 2% is the preferred acid component of the produce washing or soaking solution, other acids can be substituted therefore including, but  
20 not limited to, acetic acid, malic, propionic, tartaric, mandelic and phosphoric acid, at similar concentrations and pHs as the lactic acid.

Within the scope of this invention the term produce means fresh fruits and vegetables. Carcasses include skinned or de-haired or de-feathered carcasses of beef, hog, buffalo, game or poultry, among others. Cut meat products are cuts of meat or poultry, e.g., chicken breasts  
25 or wings or roasts or steaks, but ground meat products are not included within this definition.

A produce wash is a solution used to bathe the surface of produce, and typically is in

contact with the produce from about 30 sec to about 5 min. A produce soak is a solution in which produce items are immersed for a time from about 30 sec to about 30 min. However, the terms and solutions can be used interchangeably unless otherwise distinguished. It is understood that the temperature at which produce is washed or soaked will influence the length of time necessary to reduce or inactivate bacteria thereon, with warmer temperatures leading to a shorter time necessary for treatment.

The wash or soak solutions of the present invention can be used to reduce bacterial number, especially bacterial pathogen number, on the surfaces of fruits, vegetables, raw cut meat products, fish, shellfish, at the consumer level (in the household), in commercial food preparation environments, on fruits and/or vegetables prior to juicing, by wholesale or retail operators, and/or at the level of the harvest, meat packing plant or slaughterhouse, fishing boat, and so on, without limitation. The present methods are particularly useful for inactivating *E. coli*. O157:H7 on the surfaces of fresh fruits and vegetables.

All references cited in the present application are incorporated by reference herein to the extent that they are not inconsistent with the present disclosure.

The following examples are provided for illustrative purposes, and are not intended to limit the scope of the invention as claimed herein. Any variations in the exemplified sequences and methods which occur to the skilled artisan are intended to fall within the scope of the present invention.

20

## EXAMPLES

### Example 1. Test Chemicals

Stock solutions of 10 and 15% lactic acid, 10% hydrogen peroxide, and 10% sodium benzoate (Sigma Chemical Co., St. Louis, MO) were prepared in sterile distilled water and filter sterilized (0.22  $\mu$ m nylon filter, Nalgene, Rochester, NY). A stock solution of 0.5% glycerol monolaurate was prepared in 95% ethanol.

## Example 2. Bacterial Cultures and Media

Five strains of *E. coli* O157:H7 [E06 (milk isolate), E08 (meat isolate), E10 (meat isolate), E16 (meat isolate), and E22 (calf feces isolate)] were used for the study. The strains of *E. coli* O157:H7 were each cultured in 100 ml of sterile tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) in 250 ml Erlenmeyer flasks at 37°C for 24 hr with agitation (150 rpm). Following incubation, the bacteria were sedimented by centrifugation (4000 x g for 20 min), and washed and resuspended in 10 mM phosphate buffered saline (PBS, pH 7.2). The optical density of the solution was determined (OD of 0.5 at 640 nm represents approximately 10<sup>9</sup> CFU/ml, CFU are colony forming units). Each culture was diluted with 9 ml of sterile 0.1% peptone to yield an approximate bacterial population of 10<sup>8</sup> CFU/ml. The viable bacterial population in each culture was confirmed by plating 0.1 ml portions of appropriately diluted culture on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) plates and incubating the plates at 37°C for 24 hr. An approximately equal population of each of the five strains was combined and 0.1 ml of the suspension was used as the inoculum.

## Example 3. Sample Inoculation and Treatments

One ml of 10 or 15% lactic acid solution and 100 µl of 10% hydrogen peroxide solution were added to individual tubes containing 8.9 ml of 0.1% peptone water to yield solutions containing 1.5% lactic acid plus 0.1% hydrogen peroxide and 1% lactic acid plus 0.1% hydrogen peroxide, respectively. Controls were prepared by adding 1.1 ml of sterile distilled water to 8.9 ml of 0.1% peptone. To each tube, 100 µl of the five-strain mixture of *E. coli* O157:H7 (10<sup>7</sup> CFU) was added, and the treatments were incubated in a water bath (Pharmacia LKB, Piscataway, NJ) at 8°C for 0, 20 and 30 min; at 22°C for 0, 10 and 20 min; and at 40°C for 0, 5 and 10 min. Following incubation, a 1 ml portion of the inoculated treatment was serially diluted (1:10) in 9 ml of sterile 0.1% peptone water and 0.1 ml portions from appropriate dilutions were surface plated in duplicate on TSA plates. A volume of 1 ml of the inoculated solution after exposure to the treatment for 0, 5 and 10 min also was transferred to separate 250 ml Erlenmeyer flasks containing 100 ml of sterile TSB and incubated at 37°C for 24 hr. Colonies of *E. coli* O157:H7 were enumerated on TSA plates after incubation at 37°C for 24 hr. Following enrichment in TSB, the culture was streaked on Sorbitol MacConkey agar No.3 (Oxoid Division, Unipath Co., Ogdensburg, NY) and the

plates were incubated at 37°C for 24 hr. Representative sorbitol-negative colonies from the plates were then confirmed as *E. coli* O157:H7 by the *E. coli* O157:H7 latex agglutination assay (Remel Microbiology Products, Lenexa, KS) and the API-20E diagnostic test kit (Biomérieux, Hazelwood, MO).

5 Similarly, 1 ml of 10 or 15% lactic acid solution and 100  $\mu$ l of 10% sodium benzoate or 0.5% glycerol monolaurate (monolaurin) solution were added to individual tubes containing 8.9 ml of 0.1% peptone water to yield solutions containing 1.5% lactic acid plus 0.1% sodium benzoate or 0.005% glycerol monolaurate and 1% lactic acid plus 0.1% sodium benzoate or 0.005% glycerol monolaurate, respectively. To each of the tubes, 100  $\mu$ l of the  
10 five-strain mixture of *E. coli* O157:H7 ( $10^7$  CFU) was added and the solutions were incubated in a water bath at 8°C for 0, 20 and 30 min; at 22°C for 0, 10 and 20 min; and at 40°C for 0, 10 and 15 min. Following incubation, the populations of *E. coli* O157:H7 that survived in each treatment were determined according to the procedure described herein above.

At all the three incubation temperatures, the populations of *E. coli* O157:H7 that  
15 survived in 0.1% peptone solution containing solely 1.5% lactic acid or 0.1% hydrogen peroxide, or 0.1% sodium benzoate or 0.005% glycerol monolaurate also were determined. The pH of 0.1% peptone solution before and after addition of each combination of chemicals was determined (pH  
meter model 350, Corning Inc., Corning, NY). At least duplicate samples were assayed for  
20 each treatment and the entire study was replicated four times.

#### Example 4. Statistical Analysis

For each treatment, the data from the independent replicate trials were pooled and the mean value with standard deviation was determined [Steele and Torrie (1980) Principles and Procedures of Statistics. McGraw Hill, New York].

#### 25 Example 5. Fresh Fruit Model Testing

The efficacy of the combination of lactic acid with hydrogen peroxide for inactivating *Escherichia coli* O157:H7 on apples and oranges was tested.



Five strains of *E. coli* O157:H7 (E-06, E-08, E-10, E-16, and E-22) were used for the study. Each strain was grown individually in 100 ml of tryptic soy broth (TSB, Difco, Detroit, MI) at 37°C with agitation (150 rpm) for 20 h. Following incubation, the bacteria (100 ml) were sedimented by centrifugation (4,000 x g for 30 min) in separate tubes and washed and resuspended in 10 ml of sterile 0.1% peptone water. The five strains of the pathogen (10 ml each) were combined and used as the inoculum. The bacterial population in the suspension was determined by plating 0.1 ml of appropriate dilutions of the suspension on duplicate Sorbitol MacConkey agar plates (SMA, Oxoid Division, Unipath Co., NY) and incubating the plates at 37°C for 24 h.

Apples and oranges were inoculated and treated according to the flowchart in Fig. 5. Red Delicious apples and navel oranges were purchased from a local grocery store.

For each experiment, a total of nine apples or oranges were washed in water containing 0.15% crystal clear residue remover (Bonagra Technologies and Services, CA), scrubbed and rinsed with deionized water to remove the external wax coating on their surfaces. After washing, the apples and oranges were dried with a paper towel. The fruits were placed on a sterile plastic tray and 100 µl of *E. coli* O157:H7 culture (10<sup>8</sup> CFU) was applied near the stem end of the fruit as shown in Figure 6. The inoculated fruits were dried in a laminar flow hood for 1 h at room temperature (23°C). After drying, the populations of *E. coli* O157:H7 on three apples or oranges were determined (baseline). From the remaining six fruits, three of them (inoculated stem end) were held immersed in 50 ml of sterile deionized water containing 1.5% lactic acid plus 1.5% hydrogen peroxide at 40°C for 5, 10 or 15 min. (treatment). Simultaneously, the remaining three apples or oranges were immersed in sterile deionized water at 40°C for the same durations specified above (control). Immediately after treatments, each apple or orange was transferred to a separate, sterile, 500 ml "Whirlpak" plastic bag (Nasco, Fort Atkinson, Wisconsin) containing 50 ml of sterile 0.1% peptone water and the population of *E. coli* O157:H7 was determined on fruits as described above.

The populations of *E. coli* O157:H7 on apples and oranges were determined as follows. To each bag containing 50 ml of sterile 0.1% peptone water, an apple or orange was

transferred in such a way that the inoculated stem end of the fruit was submerged in the diluent. The bag containing the fruit was sealed and placed on a bench-top orbital shaker and subjected to shaking at 400 rpm for 2 min. A 1.0 ml portion of solution from each "Whirl-pak" bag was serially diluted with 9.0 ml of sterile 0.1% peptone water and 0.1 ml portions from appropriate dilutions were plated on duplicate tryptic soy agar (TSA, Difco, Detroit, MI) plates. The colonies of bacteria were enumerated after incubating the plates at 37°C for 24 h. The population of *E. coli* O157:H7 in the 50 ml of peptone water in each bag was determined and was expressed per apple or orange. A volume of 1 ml of 0.1% peptone water from each bag also was transferred to separate 250 ml Erlenmeyer flasks containing 100 ml of sterile TSB and incubated at 37°C for 24 hr. Following enrichment in TSB, the culture was streaked on SMA and the plates were incubated at 37°C for 24 h. Representative sorbitol-negative colonies from SMA plates and colonies from TSA plates were confirmed as *E. coli* O157:H7 by *E. coli* O157:H7 latex agglutination assay (Remel Microbiology Products, Lenexa, KS). The entire experiment with each fruit was replicated three times. In the case of oranges, exposure times of only 10 and 15 min with the treatment solution or water (control) were evaluated.

Table 1. Inactivation of *E. coli* O157:H7 on apples by combination of 1.5% lactic acid + 1.5% hydrogen peroxide at 40°C.

Samples	Surviving bacterial population (mean log cfu/apple) after		
	5 min. exposure	10 min. exposure	15 min. exposure
Baseline	7.0 ± 0.05	7.0 ± 0.05	6.95 ± 0.08
Treatment	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
Control	5.80 ± 0.16	5.30 ± 0.14	5.20 ± 0.25
Treatment solution (cfu/ml)	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
Control solution (cfu/ml)	4.56 ± 0.16	4.53 ± 0.13	4.40 ± 0.37

<sup>a</sup>negative by enrichment.

Table 2. Inactivation of *E. coli* O157:H7 on oranges by combination of 1.5% lactic acid + 1.5% hydrogen peroxide at 40°C.

Samples	Surviving bacterial population (mean log cfu/orange) after	
	10 min. exposure	15 min. exposure
Baseline	7.27 ± 0.15	7.30 ± 0.13
Treatment	1.5 ± 1.00	0.90 ± 0.33
Control	5.58 ± 0.13	5.70 ± 0.11
Treatment solution (cfu/ml)	0 <sup>a</sup>	0 <sup>a</sup>
Control solution (cfu/ml)	4.51 ± 0.17	4.53 ± 0.15

<sup>a</sup>negative by enrichment.

## I CLAIM:

1. A method for reducing bacterial numbers on a fresh food item suspected of being contaminated with at least one foodborne pathogen, said method comprising the step of contacting the fresh food item in a decontaminating solution comprising a first  
5 active ingredient, wherein said first active ingredient is an acid in a concentration from about 0.3 to about 5 percent (volume/volume) selected from the group consisting of lactic, acetic, malic, propionic, tartaric, mandelic, and phosphoric acid. and a second active ingredient selected from the group consisting of hydrogen peroxide at a concentration of from about 0.1% to 5% percent, glycerol monolaurate  
10 at a concentration from about 0.0001% to about 0.1 percent (vol/vol) and sodium benzoate at a concentration of from about 0.005 to about 0.5 percent,  
  
wherein the fresh food item is in contact with the decontaminating solution for a time and at a temperature sufficient to reduce the numbers of at least one foodborne pathogen at least 1000-fold.
- 15 2. The method of claim 1 wherein said first active ingredient is lactic acid at a concentration of from 1 to about 2 percent lactic acid and wherein said second active ingredient is hydrogen peroxide at a concentration of from about 0.1 to about 2 percent.
- 20 3. The method of claim 1 wherein said first active ingredient is lactic acid at a concentration of about 1.5 percent and wherein said second active ingredient is hydrogen peroxide at a concentration of about 1.5 percent.
4. The method of claim 1 wherein said first active ingredient is lactic acid at a concentration of from about 1 to about 2 percent and wherein said second active ingredient is sodium benzoate at a concentration from about 0.05 to about 0.5 percent.
- 25 5. The method of claim 4 wherein said first active ingredient is lactic acid at a

concentration between 1. and 2 percent and wherein said second active ingredient is sodium benzoate at a concentration of 0.1 percent.

6. The method of claim 1 wherein said first active ingredient is lactic acid at a concentration from about 1 to about 2 percent and wherein said second active ingredient is glycerol monolaurate at a concentration of 0.001 to 0.05 percent.
7. The method of claim 1 wherein the foodborne pathogen suspected of contaminating the fresh food item is *Escherichia coli* O157:H7.
8. The method of claim 1 wherein the contacting of the decontaminating solution and the fresh food item is for about 30 seconds to about 30 minutes.
9. The method of claim 1 wherein the contacting of the decontaminating solution and the fresh food item is at a temperature from about 4 to about 50° C.
10. The method of claim 1 wherein the fresh food item to be contacted with the decontaminating solution is a fruit or a vegetable.

**AMENDED CLAIMS**

[received by the International Bureau on 28 June 1999 (28.06.99);  
original claim 1 amended; remaining claims unchanged (1 page)]

1. A method for reducing bacterial numbers on a fresh food item suspected of being contaminated with at least one foodborne pathogen, said method comprising the step of contacting the fresh food item in a decontaminating solution comprising a first  
5 active ingredient, wherein said first active ingredient is an acid in a concentration from about 0.3 to about 5 percent (volume/volume) selected from the group consisting of lactic, acetic, malic, propionic, tartaric, mandelic, and a second active ingredient selected from the group consisting of hydrogen peroxide at a concentration of from about 0.1% to 5% percent, glycerol monolaurate at a concentration from about  
10 0.0001% to about 0.1 percent (vol/vol) and sodium benzoate at a concentration of from about 0.005 to about 0.5 percent,

wherein the fresh food item is in contact with the decontaminating solution for a time and at a temperature sufficient to reduce the numbers of at least one foodborne pathogen at least 1000-fold.

- 15 2. The method of claim 1 wherein said first active ingredient is lactic acid at a concentration of from 1 to about 2 percent lactic acid and wherein said second active ingredient is hydrogen peroxide at a concentration of from about 0.1 to about 2 percent.
- 20 3. The method of claim 1 wherein said first active ingredient is lactic acid at a concentration of about 1.5 percent and wherein said second active ingredient is hydrogen peroxide at a concentration of about 1.5 percent.
4. The method of claim 1 wherein said first active ingredient is lactic acid at a concentration of from about 1 to about 2 percent and wherein said second active ingredient is sodium benzoate at a concentration from about 0.05 to about 0.5 percent.
- 25 5. The method of claim 4 wherein said first active ingredient is lactic acid at a

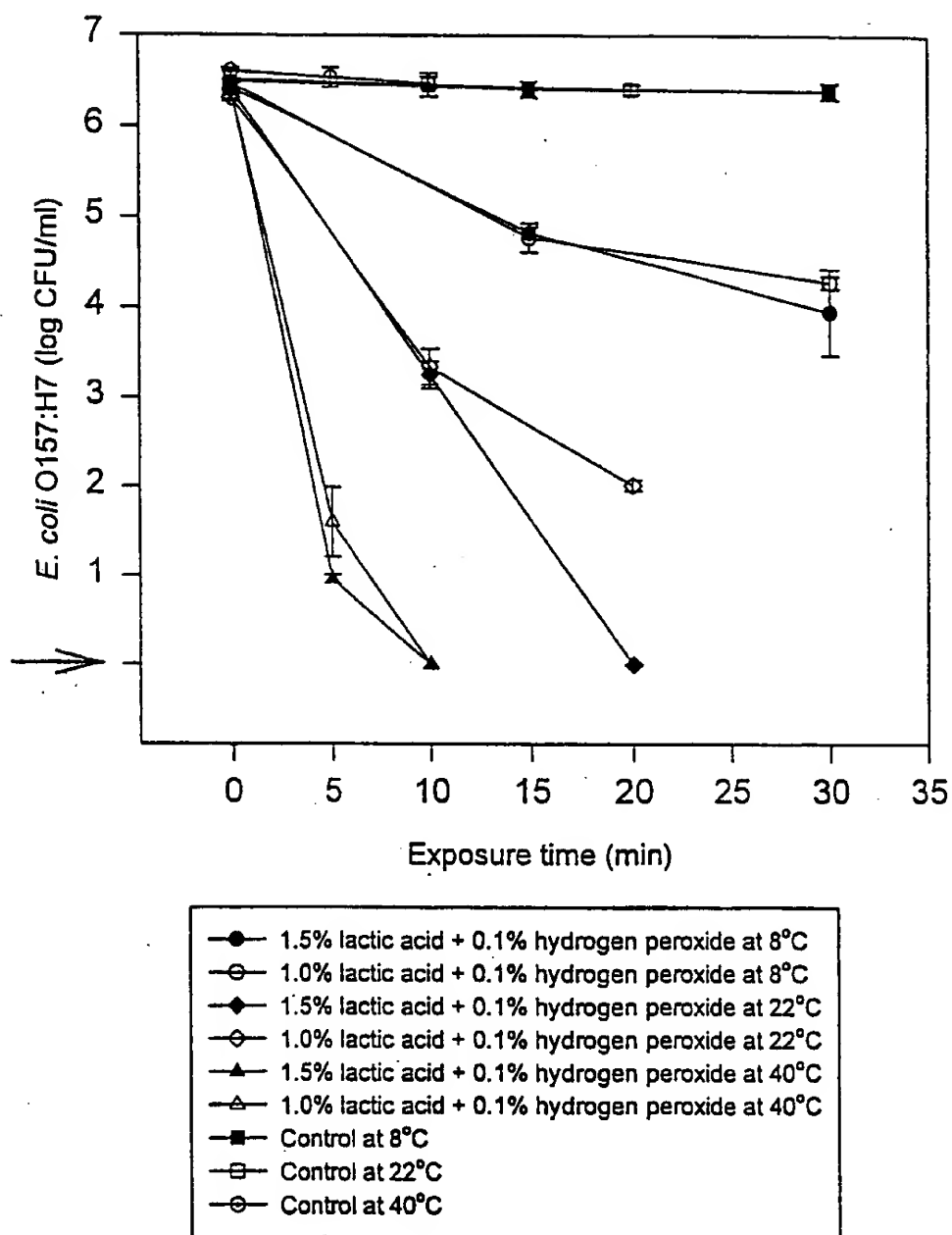
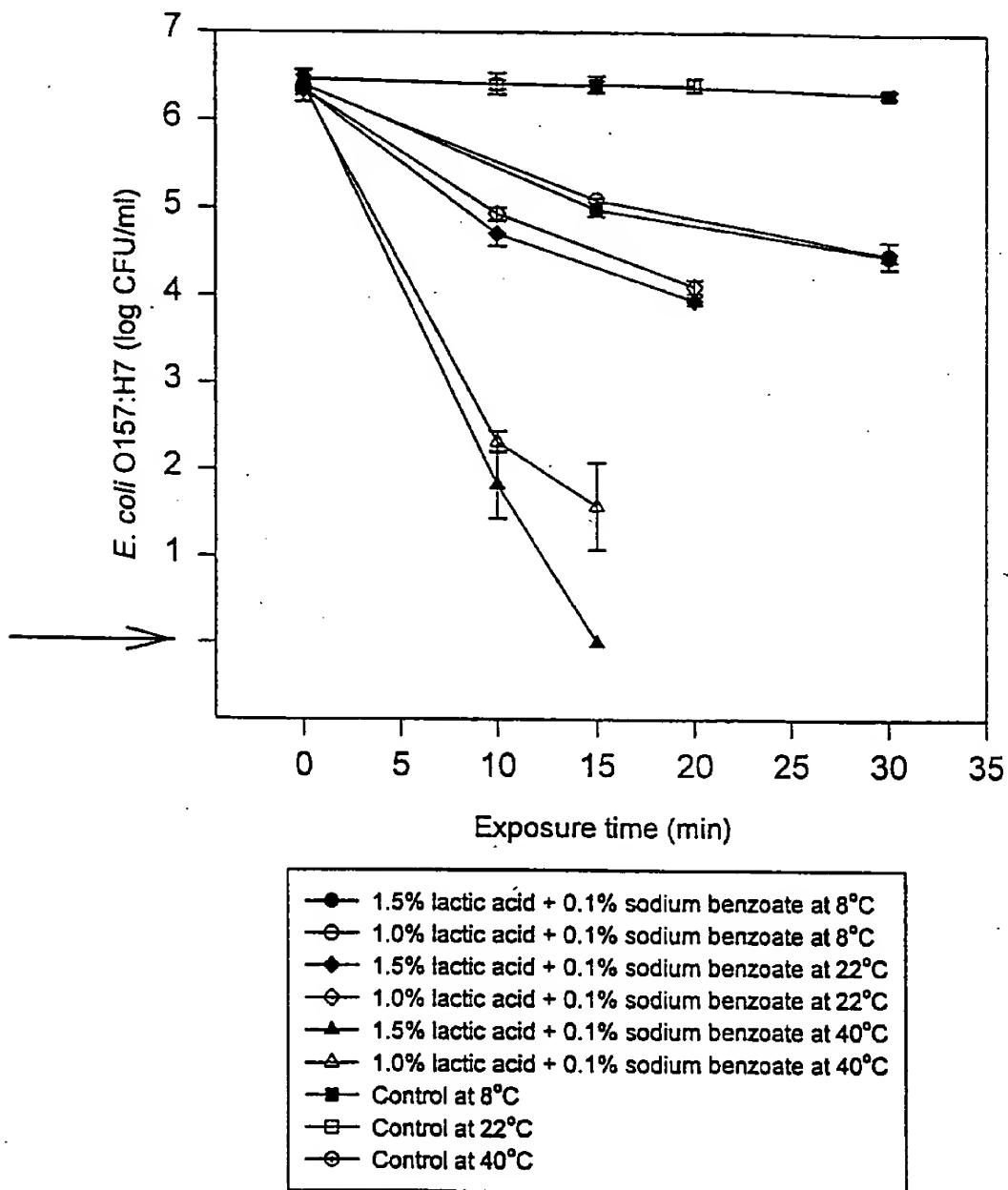
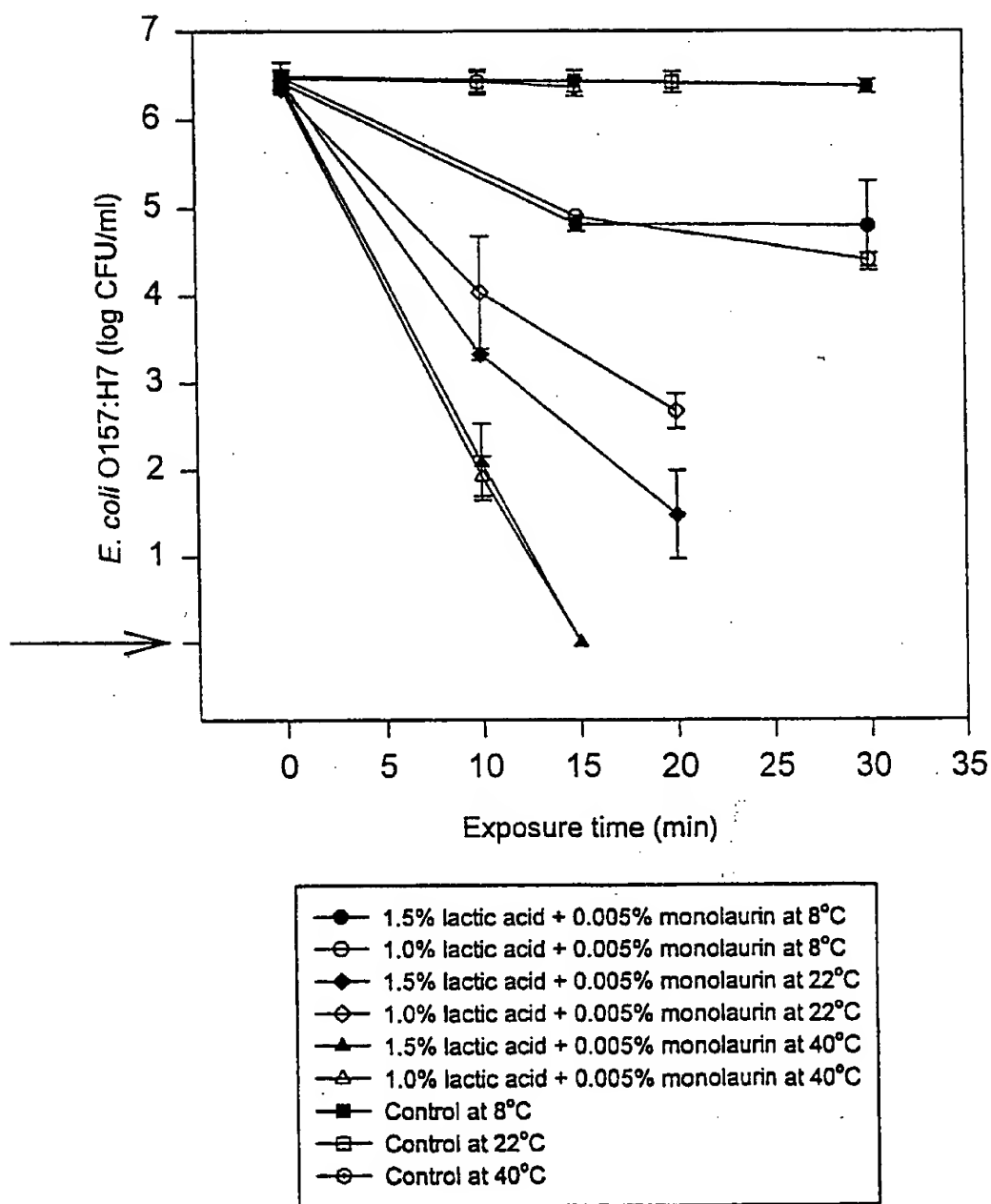
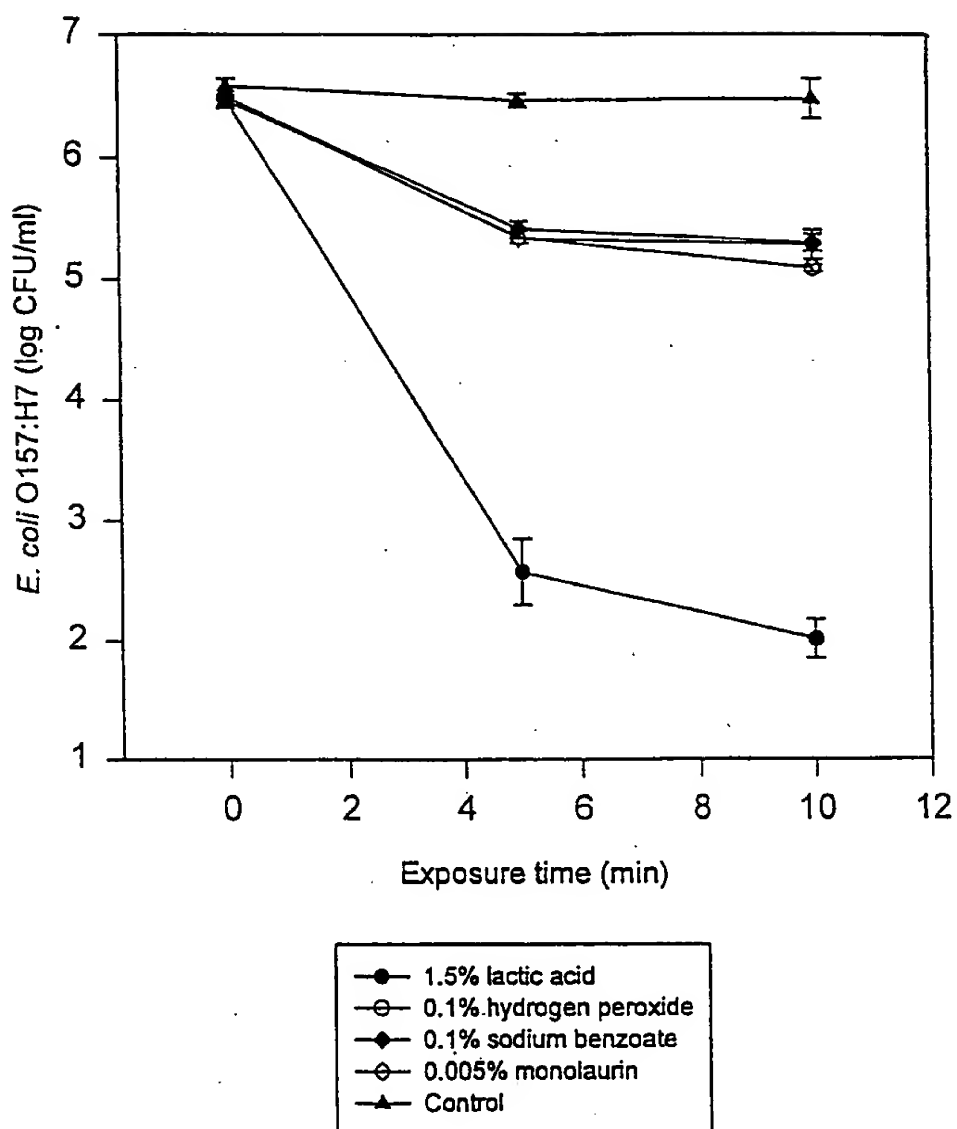


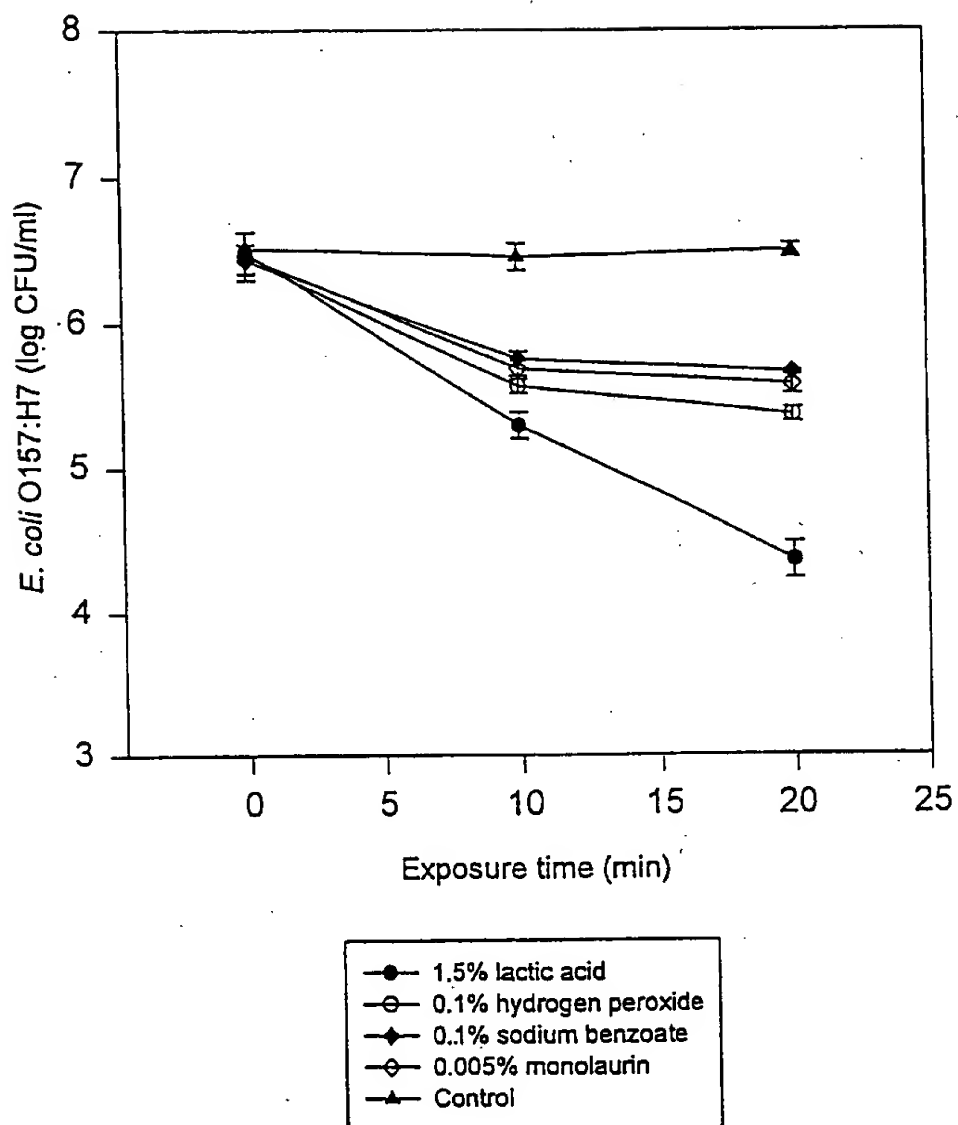
FIG. 1

**FIG. 2**

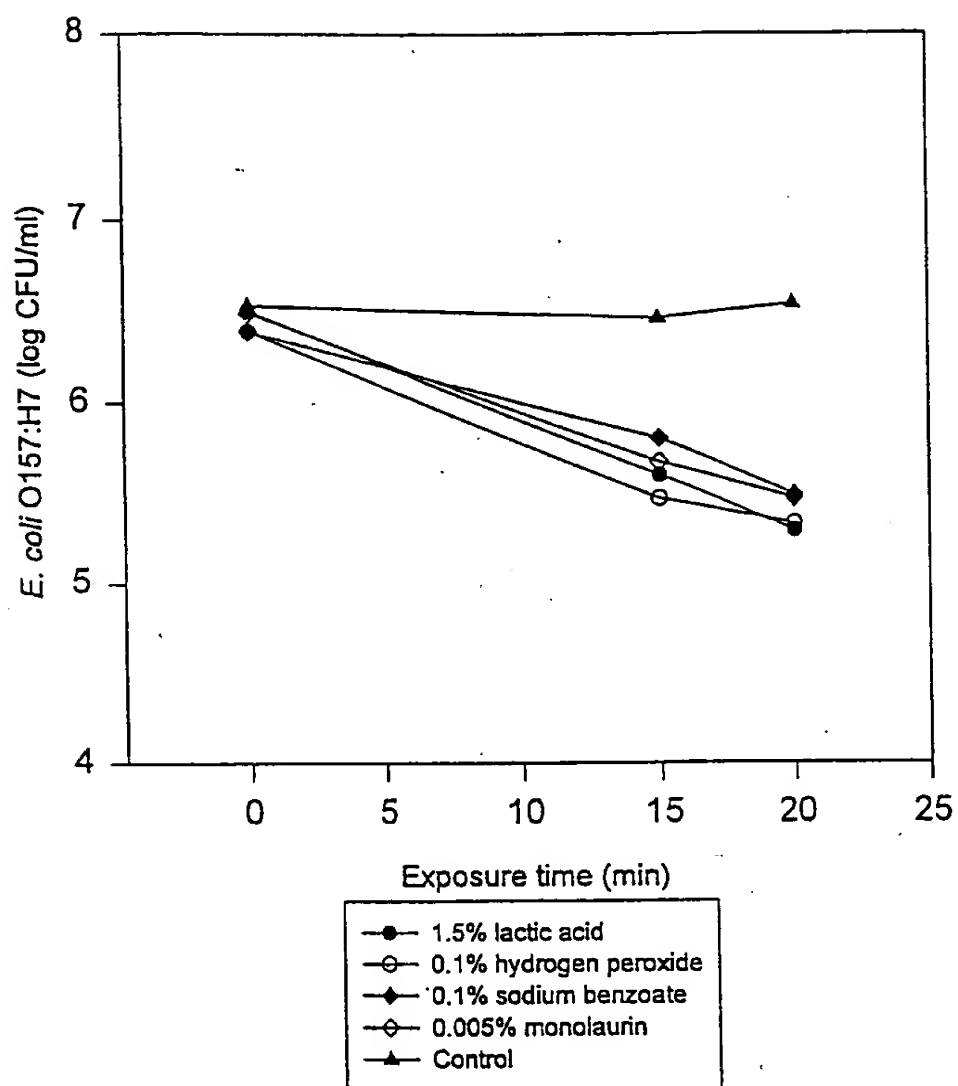


**FIG. 3**

**FIG. 4A**

**FIG. 4B**

6/8

**FIG. 4C**

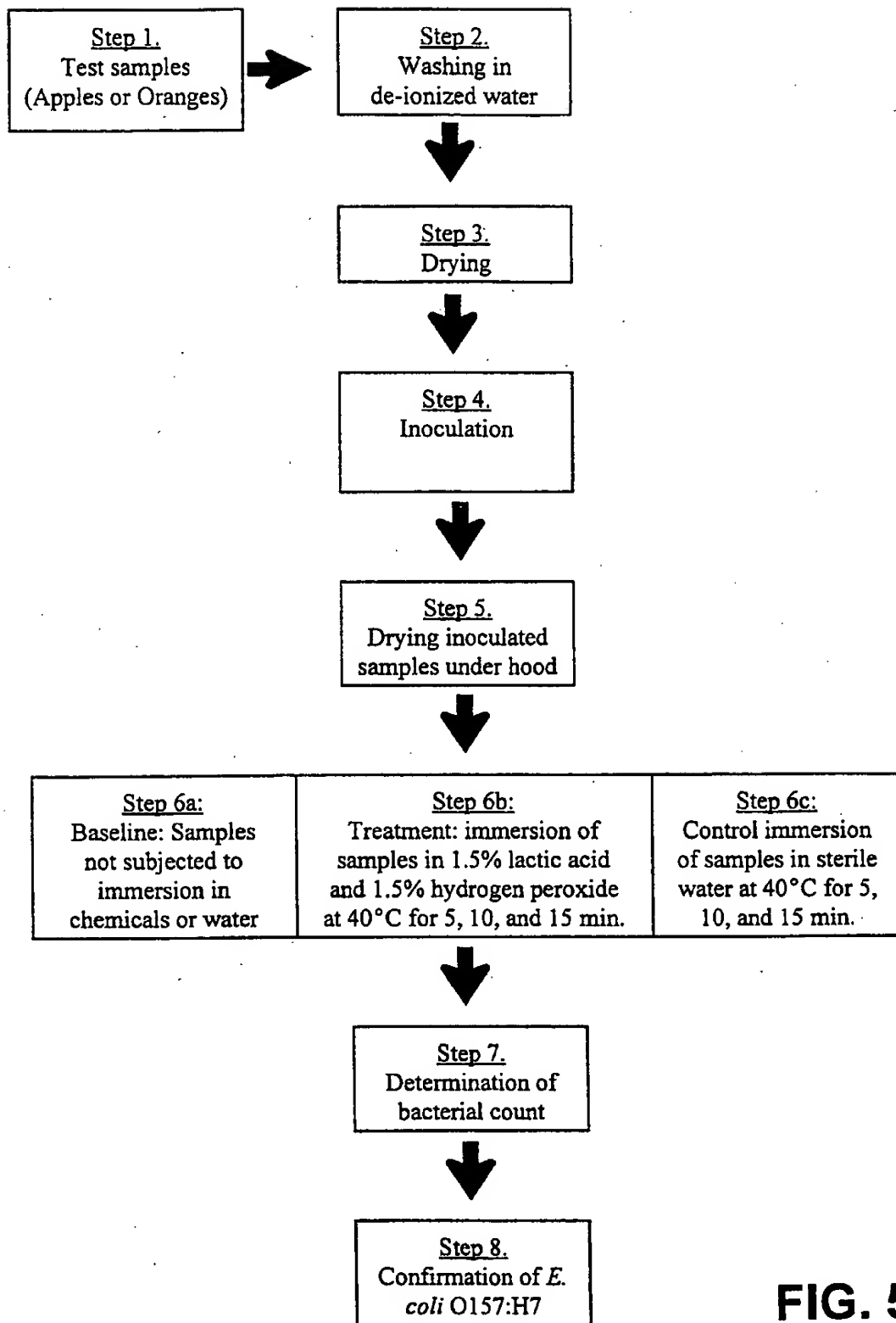
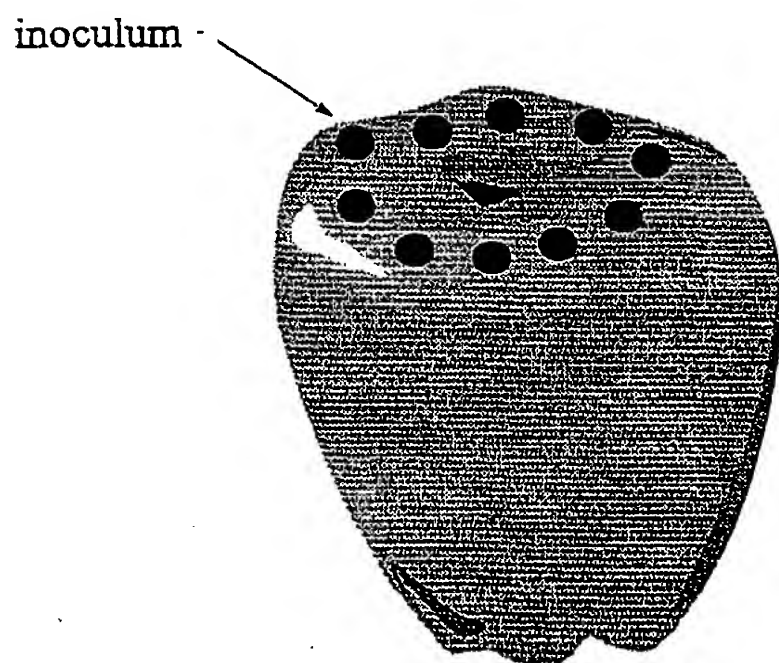


FIG. 5



**FIG. 6**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/04586**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A23L 3/34

US CL :426/321, 331, 335, 532, 652

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 426/321, 331, 335, 532, 652

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: disinfectant, decontaminate, hydrogen peroxide, food, acid, glycerol monolaurate

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,641,530 A (CHEN) 24 June 1997 (24-06-97), abstract and col. 4 ; lines 1-45, and col. 5, lines 1-45.	1-5, 6-10
Y	US 5,460,833 A (ANDREWS et al.) 24 October 1995 (24-10-95), abstract and col. 2, lines 30-70.	1, 6
Y	US 5,490,992 A (ANDREWS et al.) 13 February 1996 (13-02-96), abstract and col. 3, lines 25-70.	1, 6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 APRIL 1999

Date of mailing of the international search report

25 MAY 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

HELEN PRATT

Telephone No. (703) 308-0651

**THIS PAGE BLANK (USPTO)**